

PCT

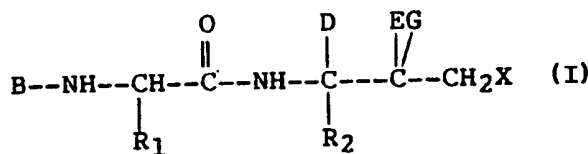
WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 37/00, 37/02 C07C 229/06, 381/02 C07D 265/30		A1	(11) International Publication Number: WO 94/04172 (43) International Publication Date: 3 March 1994 (03.03.94)
(21) International Application Number: PCT/US93/07767 (22) International Filing Date: 18 August 1993 (18.08.93) (30) Priority data: 07/932,791 20 August 1992 (20.08.92) US (71) Applicant: PROTOTEK, INC. [US/US]; 6747 Sierra Court, Dublin, CA 94568 (US). (72) Inventors: Zimmerman, Mary, P. ; 3727 Crofter's Court, Pleasanton, CA 94588 (US). BISSELL, Eugene, R. ; 101 Via Lucia, Alamo, CA 94507 (US). SMITH, Robert, E. ; 574 Escondido Circle, Livermore, CA 94550 (US).			(74) Agents: McNETT, John, C. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.

(54) Title: PEPTIDYL ACTIVATED KETONE INHIBITORS CONTAINING NATURAL AND UNNATURAL PEPTIDE SEQUENCES



(57) Abstract

A method of treating medical conditions with proteinase inhibitors of formula (I), wherein B is H or an amino acid blocking group for the N-terminal amino acid nitrogen; R₁ is an optionally protected α-amino acid side chain such that P₂ is the residue of an α-amino acid selected from the group consisting of phenylalanine (Phe), leucine (Leu), tyrosine (Tyr) and valine (Val), and substituted analogues thereof, particularly including Tyr(OMe); R₂ is an optionally protected α-amino acid side chain such that P₁ is the residue of an α-amino acid selected from the group consisting of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His), homophenylalanine (HPhe), phenylalanine (Phe), ornithine (Orn), serine (Ser) and threonine (Thr), and substituted analogues thereof; X is a fluorine-free leaving group selected from the group consisting of phenoxy, substituted phenoxy and heterophenoxy; E and G are one or more atoms more electronegative than carbon; and D is hydrogen, methyl or a substituted methyl.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NE	Niger
BE	Belgium	GN	Guinea	NL	Netherlands
BF	Burkina Faso	GR	Greece	NO	Norway
BG	Bulgaria	HU	Hungary	NZ	New Zealand
BJ	Benin	IE	Ireland	PL	Poland
BR	Brazil	IT	Italy	PT	Portugal
BY	Belarus	JP	Japan	RO	Romania
CA	Canada	KP	Democratic People's Republic of Korea	RU	Russian Federation
CF	Central African Republic	KR	Republic of Korea	SD	Sudan
CG	Congo	KZ	Kazakhstan	SE	Sweden
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovak Republic
CM	Cameroon	LJ	Luxembourg	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	MC	Monaco	TC	Togo
CZ	Czech Republic	MG	Madagascar	UA	Ukraine
DE	Germany	ML	Mali	US	United States of America
DK	Denmark	MN	Mongolia	UZ	Uzbekistan
ES	Spain			VN	Viet Nam
FI	Finland				

PEPTIDYL ACTIVATED KETONE INHIBITORS
CONTAINING NATURAL AND UNNATURAL PEPTIDE SEQUENCES

BACKGROUND OF THE INVENTION

The present invention relates generally to peptidyl
5 ketone inhibitors, and more particularly to peptidyl ketone
inhibitors designed for the in vivo management of cysteine
proteases, particularly cathepsin B and L, and their
primitive enzymatic counterparts.

Cathepsins B and L are cysteinyl proteases involved in
10 normal protein degradation. As such, they are generally
located in the lysosomes of cells. When these enzymes are
found extralysosomally they have been implicated by use of
synthetic substrate technology and by natural endogenous
inhibitors as playing a causative role in a number of disease
15 states such as rheumatoid arthritis, osteo arthritis,
pneumocystis carinii, schistosomiasis, trypanosoma cruzi,
trypanosoma brucei brucei, Crithidia fusiculata, malaria,
periodontal disease, tumor metastasis, metachromatic
leukodystrophy, muscular dystrophy, etc. In particular, a
20 connection between cathepsin B-type enzymes and rheumatoid
arthritis has been suggested in van Noorden and Everts,
"Selective Inhibition of Cysteine Proteinases by
Z-Phe-Ala-CH₂F Suppresses Digestion of Collagen by
Fibroblasts and Osteoclasts," 178 Biochemical and Biophysical
25 Research Communications 178; Rifkin, Vernillo, Kleekner,
Auszmann, Rosenberg and Zimmerman, "Cathepsin B and L
Activities in Isolated Osteoclasts," 179 Biochemical and
Biophysical Research Communications 63; Grinde, "The Thiol
Proteinase Inhibitors, Z-Phe-Phe-CHN₂ and Z-Phe-Ala-CHN₂,
30 Inhibit Lysosomal Protein Degradation in Isolated Rat
Hepatocytes," 757 Biochimica et Biophysica Acta 15; Mason,
Bartholomew and Hardwick, "The Use of

-2-

Benzyloxycarbonyl[¹²⁵I]iodotyrosylalanyldiazomethane as a Probe for Active Cysteine Proteinases in Human Tissues," 263 Biochem. J. 945; van Noorden, Smith and Rasnick, "Cysteine Proteinase Activity in Arthritic Rat Knee Joints and the Effects of a Selective Systemic Inhibitor, Z-Phe-Ala-CH₂F," 15 J. Rheumatol. 1525; and van Noorden, Vogels and Smith, "Localization and Cytophotometric Analysis of Cathepsin B Activity in Unfixed and Undecalified Cryostat Sections of Whole Rat Knee Joints," 37 J. Histochemistry and Cytochemistry 617. A connection between cathepsin B and osteo arthritis has been suggested in Delaissé, Eeckhout and Vaes, "In Vivo and In Vitro Evidence for the Involvement of Cysteine Proteinases in Bone Resorption," 125 Biochemical and Biophysical Research Communications 441; a connection between cathepsin B and pneumocystis carinii has been suggested in Hayes, Stubberfield, McBride and Wilson, "Alterations in Cysteine Proteinase Content of Rat Lung Associated with Development of Pneumocystis Carinii Infection," 59 Infection and Immunity 3581; a connection between cysteine proteinases and schistosomiasis has been suggested in Cohen, Gregoret, Amiri, Aldape, Railey and McKerrow, "Arresting Tissue Invasion of a Parasite by Protease Inhibitors Chosen With the Aid of Computer Modeling," 30 Biochemistry 11221. A connection between cysteine proteinases and trypanosoma cruzi, trypanosoma brucei brucei and crithidia fasciculata has been suggested in Ashall, Harris, Roberts, Healy and Shaw, "Substrate Specificity and Inhibitor Sensitivity of a Trypanosomatid Alkaline Peptidase," 1035 Biochimica et Biophysica Acta 293, and/or in Ashall, Angliker and Shaw, "Lysis of Trypanosomes by Peptidyl Fluoromethyl Ketones," 170 Biochemical and Biophysical Research Communications 923. A connection between cysteine proteinases and malaria has been suggested in Rosenthal, Wollish, Palmer and Rasnick, "Antimalarial Effects of Peptide Inhibitors of a Plasmodium Falciparum Cysteine Proteinase," 88 J. Clin. Invest. 1467,

-3-

- and in Rosenthal, Lee and Smith, "Inhibition of a Plasmodium Vinckei Cysteine Proteinase Cures Murine Malaria," (in press). A connection between cathepsin B and tumor metathesis has been suggested in Smith, Rasnick, Burdick,
- 5 Cho, Rose and Vahratian, "Visualization of Time-Dependent Inactivation of Human Tumor Cathepsin B Isozymes by a Peptidyl Fluoromethyl Ketone Using a Fluorescent Print Technique," 8 Anti-cancer Research 525. A connection between cathepsin B and cancer has been suggested in Gordon and
- 10 Mourad, 2 Blood Coagulation and Fibrinolysis 735. A connection between cathepsin B and periodontal disease has been suggested in Cox, Cho, Eley and Smith, "A Simple, Combined Fluorogenic and Chromogenic Method for the Assay of Proteases in Gingival Crevicular Fluid," 25 J. Periodont. Res. 164; Uitto, Larjava, Heino and Sorsa, "A Protease of Bacteroides Gingivalis Degrades Cell Surface and Matrix Glycoproteins of Cultured Gingival Fibroblasts and Induces Secretion of Collagenase and Plasminogen Activator," 57 Infection and Immunity 213; Kunitatsu, Yamamoto, Ichimaru,
- 20 Kato and Kato, "Cathepsins B, H and L Activities in Gingival Crevicular Fluid From Chronic Adult Periodontitis Patients and Experimental Gingivitis Subjects," 25 J Periodont Res 69; Beighton, Radford and Naylor, "Protease Activity in Gingival Crevicular Fluid From Discrete Periodontal Sites in Humans With Periodontitis or Gingivitis"; 35 Archs oral Biol. 329; Cox and Eley, "Preliminary Studies on Cysteine and Serine Proteinase Activities in Inflamed Human Gingiva Using Different 7-Amino-4-Trifluoromethyl Coumarin Substrates and Protease Inhibitors," 32 Archs oral Biol. 599; and
- 30 Eisenhauer, Hutchinson, Javed and McDonald, "Identification of a Cathepsin B-Like Protease in the Crevicular Fluid of Gingivitis Patients," 62 J Dent Res 917. A connection between cathepsin B and metachromatic leukodystrophy has been suggested in von Figura, Steckel, Conary, Hasilik and Shaw,
- 35 "Heterogeneity in Late-Onset Metachromatic Leukodystrophy.

-4-

Effect of Inhibitors of Cysteine Proteinases," 39 Am J Hum Genet. 371; a connection between cathepsin B and muscular leukodystrophy has been suggested in Valentine, Winand, Pradhan, Moise, de Lahunta, Kornegay and Cooper, "Canine X-Linked Muscular Dystrophy as an Animal Model of Duchenne Muscular Dystrophy: A Review," 42 Am J Hum Genet. 352; a connection between cathepsin B and rhinovirus has been suggested in Knott, Orr, Montgomery, Sullivan and Weston, "The Expression and Purification of Human Rhinovirus Protease 3C," 182 Eur. J. Biochem. 547; a connection between cathepsin B and kidney disease has been suggested in Baricos, O'Connor, Cortez, Wu and Shah, "The Cysteine Proteinase Inhibitor, E-64, Reduces Proteinuria in an Experimental Model of Glomerulonephritis," 155 Biochemical and Biophysical Research Communications 1318; and a connection between cathepsin B and multiple sclerosis has been suggested in Dahlman, Rutschmann, Kuehn and Reinauer, "Activation of the Multicatalytic Proteinase from Rat Skeletal Muscle by Fatty Acids or Sodium Dodecyl Sulphate," 228 Biochem. J. 171.

Although a number of cysteine proteinase inhibitors have been identified, most of these have drawbacks for in vivo use. In particular, drawbacks such as reversibility of inhibition, lack of specificity, and rapid clearance from the body have been associated with prior art inhibitors. The microbial products antipain and leupeptin, for example, are effective but reversible inhibitors of cysteine proteinase (McConnell et al., 33 J. Med. Chem. 86-93; Sutherland et al. 110 Biochem. Biophys. Res. Commun. 332-38), and also inhibit certain serine proteinases (Umezawa, 45 Meth. Enzymol. 678-95). The compound E64 and its synthetic analogues are more selective inhibitors (see, e.g., Barret et al., 201 Biochem. J. 189-98, and Grinde, 701 Biochem. Biophys. Acta. 328-33), but disappear too quickly from the circulation for in vivo use (Hashida et al. 91 J. Biochem. 1373-80).

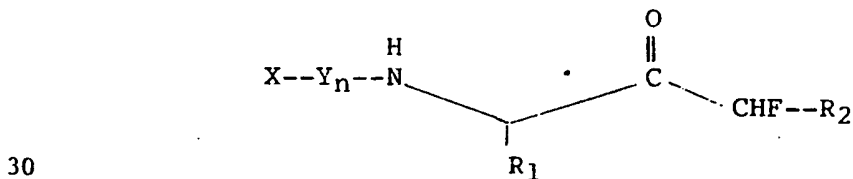
The most promising type of cysteine proteinase inhibitors

-5-

have an activated carbonyl with a suitable α -leaving group fused to a programmed peptide sequence that specifically directs the inhibitor to the active site of the targeted enzyme. Once inside the active site, the inhibitor carbonyl
 5 is attacked by a cysteine thiolate anion to give the resulting hemiacetal. If the α -leaving group then breaks off, the bond between enzyme and inhibitor becomes permanent and the enzyme is irreversibly inactivated.

The usefulness of an inhibitor in inactivating a
 10 particular enzyme therefore depends not only on the "lock and key" fit of the peptide portion, but also on the reactivity of the bond holding the α -leaving group to the rest of the inhibitor. It is important that the leaving group be reactive only to the intramolecular displacement via a
 15 1,2-migration of sulfur in the breakdown of the hemithioacetal intermediate.

Groundbreaking work regarding cysteine proteinase inhibitors having an activated carbonyl, a suitable α -leaving group and a peptide sequence effective to
 20 specifically direct the inhibitor to the active site of the targeted enzyme was disclosed in U.S. Patent 4,518,528 to Rasnick, incorporated herein by reference. That patent established peptidyl fluoromethyl ketones to be unprecedented inhibitors of cysteine proteinase in selectivity and
 25 effectiveness. The fluoromethyl ketones described and synthesized by Rasnick included those of the formula:



wherein R_1 and R_2 are independently selected from the group hydrogen, alkyl of 1-6 carbons, substituted alkyl of

-6-

1-6 carbons, aryl, and alkylaryl where the alkyl group is of 1-4 carbons; n is an integer from 1-4 inclusive; X is a peptide end-blocking group; and Y is an amino acid or peptide chain of from 1-6 amino acids.

5 Peptidylketone inhibitors using a phenol leaving group are similar to the peptidyl fluoroketones. As is known in the art, oxygen most closely approaches fluorine in size and electronegativity. Further, when oxygen is bonded to an aromatic ring these values of electronegativity become even
10 closer due to the electron withdrawing effect of the sp² carbons. The inductive effect of an α -ketophenol versus an α -ketofluoride when measured by the pKa of the α -hydrogen, appears comparable within experimental error.

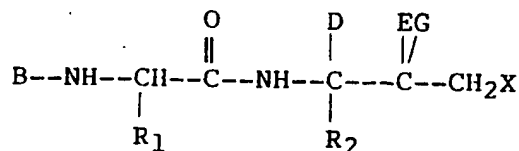
Although the general association between various disease
15 states and cysteine proteinase inhibitors is known, the prior art has not identified specific methods of treatment using specific inhibitors of the type first described by Rasnick. A need therefore exists for methods of treating various disease states characterized by extralysosomal cathepsin B
20 and/or cathepsin L activity. The present invention addresses that need.

SUMMARY OF THE INVENTION

Briefly describing the present invention, there is provided a method of treating medical conditions which are characterized by extralysosomal cathepsin B and/or cathepsin L activity. In particular, a group of cathepsin B and/or cathepsin L inhibitors which have been shown to be particularly effective in in vivo applications is disclosed.

Concerning the specific compounds employed in the present invention, preferred compounds have the formula:

10



wherein

B is H or an amino acid blocking group for the N-terminal amino acid nitrogen;

R_1 is an optionally protected α -amino acid side chain on the P_2 amino acid residue, and is selected such that the P_2 amino acid residue is the residue of an α -amino acid selected from the group consisting of phenylalanine (Phe), leucine (Leu), tyrosine (Tyr) and valine (Val), and substituted analogues thereof, particularly including Tyr(OMe);

R_2 is an optionally protected α -amino acid side chain on the P_1 amino acid residue, and is selected such that the P_1 amino acid residue is the residue of an α -amino acid selected from the group consisting of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His), homophenylalanine (HPhe), phenylalanine (Phe), ornithine (Orn), serine (Ser) and threonine (Thr), and substituted analogues thereof;

-8-

X is a fluorine-free leaving group selected from the group consisting of phenoxy, substituted phenoxy and heterophenoxy;

5 E and G are atoms more electronegative than carbon, wherein E and G may be the same atom, as in a carbonyl or hydrazone, bonded to the peptide chain by a double bond, or two separate atoms each bonded to the peptide chain by a single bond; and

D is hydrogen, methyl or a substituted methyl.

10 One object of the present invention is to provide a method of treating medical conditions which are characterized by extralysosomal cathepsin B and/or cathepsin L.

A further object of the present invention is to provide a group of cathepsin B and/or cathepsin L inhibitors which are
15 particularly effective for in vivo applications.

Further objects and advantages of the present invention will be apparent from the following description.

DESCRIPTION OF THE PREFERRED EMBODIMENT

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that
5 no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as
10 would normally occur to one skilled in the art to which the invention relates.

The present invention relates to methods of treating medical conditions which are characterized by extralysosomal cathepsin B and/or cathepsin L. In one aspect of the
15 invention, a group of cathepsin B and/or cathepsin L inhibitors which have been shown to be particularly effective in in vivo applications is disclosed.

The inhibitors of the present invention can be described generally by the formula:



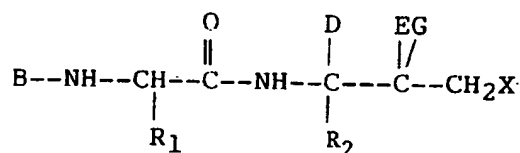
wherein

B is an amino acid blocking group for the N-terminal amino acid,
each $(P)_x$ is an optionally protected α -amino acid
25 residue, and
X is a leaving group.

As is conventional in the art, and as used herein, amino acid residues may be designated as P_1 , P_2 , etc., wherein P_1 refers to the amino acid residue nearest the leaving
30 group, P_2 refers to the amino acid residue next to P_1 and nearer the blocking group, etc. In dipeptide inhibitors therefore, P_2 is the amino acid residue nearest the blocking group.

-10-

More specifically, the compounds employed in the present invention are of the formula:



wherein

B is H or an amino acid blocking group for the N-terminal amino acid nitrogen;

R_1 is an optionally protected α -amino acid side chain on the P_2 amino acid residue, and is selected such that the P_2 amino acid residue is the residue of an α -amino acid selected from the group consisting of phenylalanine (Phe), leucine (Leu), tyrosine (Tyr) and valine (Val), and substituted analogues thereof, particularly including Tyr(OMe);

R_2 is an optionally protected α -amino acid side chain on the P_1 amino acid residue, and is selected such that the P_1 amino acid residue is the residue of an α -amino acid selected from the group consisting of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His), homophenylalanine (HPhe), phenylalanine (Phe), ornithine (Orn), serine (Ser) and threonine (Thr), and substituted analogues thereof;

X is a fluorine-free leaving group selected from the group consisting of phenoxy, substituted phenoxy and heterophenoxy;

E and G are atoms more electronegative than carbon, wherein E and G may be the same atom, as in a carbonyl or hydrazone, bonded to the peptide chain by a double bond, or two separate atoms each bonded to the peptide chain by a single bond; and

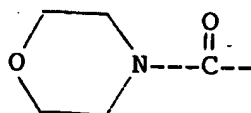
D is hydrogen, methyl or a substituted methyl.

Concerning the amino acid blocking group B for the

-11-

N-terminal amino acid nitrogen, many suitable peptide end-blocking groups are known in the art. For example the end-blocking groups identified in E. Gross and J. Meienhofer (eds.), The Peptides, Vol. 3 are generally suitable for use in the present invention. Preferred blocking groups include N-morpholine carbonyl and derivatives of propionic acid derivatives that have intrinsic analgesic or anti-inflammatory action. Examples of blocking groups having intrinsic analgesic or anti-inflammatory action may be found in Gilman, Goodman, Gilman, The Pharmacological Basis of Therapeutics, Sixth Ed. MacMillan, Chapter 29. As defined herein, the peptide end-blocking group is attached to either an amino acid or a peptide chain.

One particularly effective blocking group is the 4-morpholinylcarbonyl blocking group shown below:



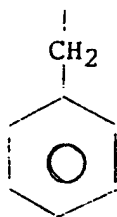
4-morpholinylcarbonyl

Concerning the side chain on the P_2 amino acid, such side chains are selected so that the linked P_2 amino acid is a member of the group consisting of phenylalanyl (Phe), leucyl (Leu), tyrosyl (Tyr) and valyl (Val) amino acid residues and substituted analogues thereof, particularly including Tyr(OMe). Extensive testing of various side chains on the P_2 amino acid has identified this group of side chains as providing superior safety and efficacy for in vivo applications such as are described herein. This superior safety and efficacy could not have been predicted from the prior art, and was established only after extensive experimentation.

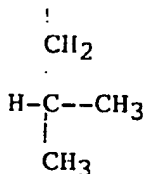
Side chains appropriate for forming such amino acid residues include:

-12-

5



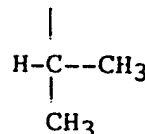
phenylalanyl



leucyl



tyrosyl



valyl

Concerning the side chain on the P_1 amino acid, such side chains are selected so that the linked P_1 amino acid is a member of the group consisting of alanyl (Ala), arginyl (Arg), aspartic acid (Asp), glutamic acid (Glu), histidyl (His), homophenylalanyl (HPhe), phenylalanyl (Phe), ornithyl (Orn), seryl (Ser) and threonyl (Thr), and optionally substituted analogues thereof such as thiazoles and amino thiazoles.

15 It is to be appreciated from the prior art that other heterocycles can replace the histidyl imidazole without a loss of potency. Further, a number of modifications to the amidine base of arginine are commonly known. Accordingly, all five- and six-membered ring heterocycles with chain
20 lengths of two to five carbon atoms and containing nitrogen, oxygen or sulfur are considered to be substituted analogues of P_1 amino acid residues for the purposes of the present invention.

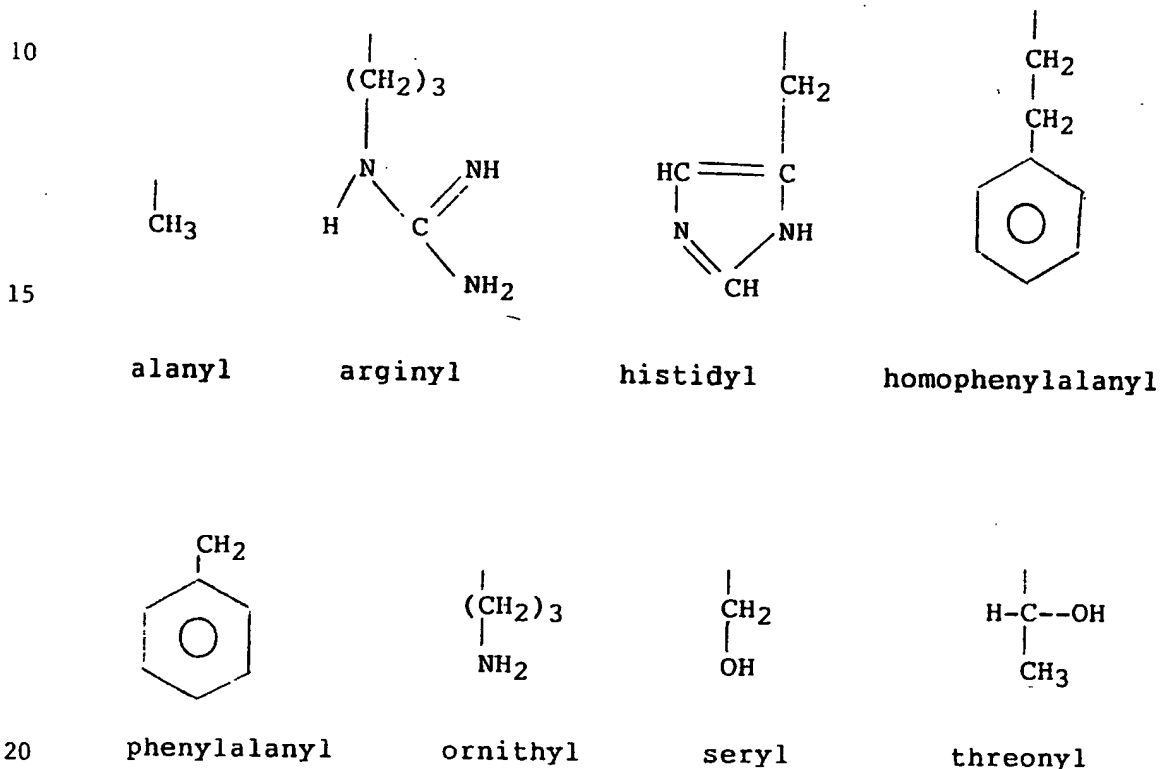
In addition, it is known that ester and amide derivatives
25 of the amino acid residues may be used without adversely affecting the suitability of the inhibitors for in vivo applications. Accordingly, such ester and amide derivatives of the above-named amino acid residues, particularly aspartic and glutamic acid residues, are intended to be included as
30 substituted analogues within the scope of the present

-13-

invention.

Again, extensive testing of various side chains on the P_1 amino acid has identified this group of side chains as providing superior safety and efficacy for in vivo applications such as are described herein. This superior safety and efficacy could not have been predicted from the prior art, and was established only after extensive experimentation.

Side chains appropriate for the P_1 amino acid include:



Concerning the leaving groups of the present invention, such leaving groups are selected from the group consisting of phenoxy, substituted phenoxy and heterophenoxy. In particular, fluorine-free leaving groups are preferred. It is to be appreciated that when using phenol as the leaving group, the pool of available phenols includes

-14-

anti-inflammatories such as aspirin and aspirin-like drugs, methyl salicylate, acetaminophen and other derivatives. Clearly, releasing such an anti-inflammatory to reduce prostoglandin production and/or pain is beneficial in certain
5 circumstances. Suitable phenoxy, substituted phenoxy and heterophenoxy leaving groups for a specific application may be selected by those skilled in the art without undue experimentation.

It is also to be appreciated that during the course of
10 the reaction of enzyme with inhibitor, the carbonyl of the inhibitor rehybridizes to sp^3 and forms a ketal intermediate with the thiol function of the enzyme. Under the acid conditions of this reaction, other ketals can exchange with this intermediate either by going through the ketone or by
15 ketal-ketal exchange. Accordingly, ketals can be substituted for carbonyls in the peptidyl inhibitors of the present invention. Similarly, other compounds such as hydrazones, hemiketals, oximes, imines, cyanohydrins, enolethers, enamines, hemithioketals, and the like are to be considered
20 carbonyl equivalents and may be substituted for a carbonyl or to give a carbonyl under the acidic conditions of these inhibition reactions. Utilization of such derivatives can also be vehicles to either improve the bioavailability of the inhibitor drug or keep it from crossing a cellular membrane
25 depending upon the hydrophobic nature of the masking function.

It is also to be appreciated that the development and synthesis of compounds having isosteric replacements of amide bonds is now a standard practice in the development of biologically active peptides once the optimum peptide
30 sequence has been identified. Accordingly, the present invention includes compounds having one or more modified amide bonds in the peptide sequence so long as conformation and binding are maintained while secondary enzymatic hydrolysis is prevented. For a list of such modifications
35 see Kaltenbronn, 33, J. Med. Chem., 838. In addition,

-15-

inhibitors having a hydrazine replacement for the P₁ nitrogen as reported by Giordano for other halogen methyl ketones are also intended to be claimed.

Example 1

5 General procedure for the preparation of 4-Morpholine carbonyl-amino acid esters. To the pTSA salt of the amino acid benzyl ester (24.12 mmol) in THF (100 ml) is added 4-morpholine carbonyl chloride (Aldrich) 24.12 mmol, followed by N-methylmorpholine (48.24 mmol). The mixture is stirred
10 at room temperature overnight, then is poured into 100 ml 1N hydrochloric acid, extracted with ethyl acetate (200 ml), washed with saturated sodium bicarbonate solution (100 ml) and brine (40 ml), dried over magnesium sulfate, filtered, and finally evaporated to give the morpholine carbonyl benzyl
15 ester. The free acid is obtained by reduction with hydrogen (30 psi), 10% palladium on carbon with ethyl acetate as the solvent.

Example 2

(4-morpholinylcarbonyl)-L-phenylalanyl-L-homophenylalanyl-
20 (2-carboxymethyl) phenoxyethyl ketone (MU-Phe-HPhe-O-Ph). To a solution of 4-morpholinecarbonyl-L-phenylalanine (8.3 mmol) in 100 ml THF at (-15°C) is added 1 equivalent (0.913 ml) N-methylmorpholine followed by isobutylchloroformate (8.3 mmol, 1.08 ml). Homophenylalanine methyl ester hydrochloride
25 salt (8.4 mol, 1.94 g) is added after 10 min of reaction followed by another equivalent (0.913 ml) of N-methylmorpholine. The reaction is allowed to come slowly to room temperature and is then stirred overnight. The reaction is poured into 100 ml HCl (1N), extracted with ethyl
30 acetate (200 ml), washed with 50 ml HCl (1N) saturated sodium bicarbonate (2X100 ml) and brine (100 ml), dried over MgSO₄, filtered and evaporated to give 3.50 g (93%) of a white foam.

-16-

The foam material is dissolved in 50 ml of methanol and 7.8 ml of 1N sodium hydroxide. After 5 hr the solution is concentrated to a gum, diluted with water and extracted with ethyl acetate. The water fraction is then neutralized with 5 1N HCl and extracted with ethyl acetate. The organic layer is dried (MgSO_4), filtered, and concentrated to dryness to give a white solid (2.65 g, 78%, m.p. 88-96°C).

General procedure for the conversion of N-blocked peptidyl free acids to N-blocked peptidyl bromomethyl ketones. One equivalent (0.495 ml, 4.5 mmol) of N-methylmorpholine is added to a solution of 4-morpholine carbonyl-phenylalanyl-homophenyl alanine (2.00 g, 4.5 mmol at -15°C). One equivalent (0.583 ml) of isobutyl chloroformate is thereafter added to the mix. The mixture is stirred at 15 this temperature for 10 min and then added through filter paper to an ethereal solution of diazomethane prepared from 4.3 g of Diazald according to the supplier's directions (Aldrich). After stirring overnight the reaction is washed with water (2X50 ml), saturated sodium bicarbonate (50 ml) 20 and brine (50 ml), dried over MgSO_4 , filtered and evaporated to dryness. Purification by column chromatography (150 g, CHCl_3 :isopropanol, 9:1) gives pure diazomethyl ketone characterized by the diazomethyl hydrogen absorption at 5.25 ppm.

25 Conversion to the bromomethyl ketone. One ml of a 30% solution of hydrogen bromide in acetic acid is diluted with 1 ml of methylene chloride and then added dropwise to a solution of 4-morpholine carbonyl-phenylalanyl-homophenyl alanyl diazo methyl ketone in 25 ml of methylene chloride at 30 -15°C. After 10 min, TLC monitoring shows a loss of starting material (R_f 0.35; silica gel, CHCl_3 :isopropanol, 9:1). The reaction is poured into 150 ml of water and extracted with ethyl acetate (2X100 ml). The combined organic fractions are washed with brine (2X50 ml), sodium bicarbonate 35 (2X50 ml) and brine again (2X50 ml), dried over Na_2SO_4

-17-

and concentrated to give 660 mg bromomethyl ketone (86% of a solid foam characterized by its NMR proton absorption at 3.82 ppm).

Preparation of phenoxymethyl ketones. Potassium fluoride (230 mg) is added to a flask containing 1 ml of DMF, 0.5 ml of methyl salicylate, and 1 g (2.2 mmol) of (4-morpholine carbonyl)-L-phenylalanyl-homophenylalanyl bromomethyl ketone. The reaction is then cooled, diluted with a solution of chloroform and isopropanol (9:1), and passed through silica gel to remove the potassium fluoride. The filtrate is evaporated to dryness and chromatographed (75 g silica gel, CH₂Cl₂:methanol, 9:1) to give 0.9 g (79%) of solid phenoxymethyl ketone, m.p. 115-121°C.

Example 3

t-Butyl carbamyl-L-arginyl-(2-Carboxymethyl) phenoxymethyl ketone. In a method according to Example 2, potassium fluoride (40 mg) is added to 42 mg of BOC-Arg-CH₂Br and methyl salicylate (800 mg). The reaction is heated to 62° under argon for 11 min, cooled, diluted with ethyl acetate, washed with water, dried over Na₂SO₄, concentrated and precipitated from ether. The product has an R_f value of 0.5 (silica gel, CHCl₃:isopropanol, 8:2) and is five times as effective as Z-Phe-Ala-FK in inhibiting cathepsin B.

Example 4

t-Butyloxycarbonyl-L-phenylalanyl-alanyl-bromomethyl ketone (BOC-Phe-Ala-bromomethyl ketone). To t-butyloxycarbonyl-L-phenylalanyl-2-carbamoyl-diazoketone (1.8 g, 5 mm) in 9 ml methylene chloride at 0°C is added in a dropwise manner 2.5 ml of 30% hydrogen bromide-acetic acid (Aldrich) which has been diluted to 5.5 ml with methylene chloride. This addition takes 2 hr at which time TLC shows the starting material to be lost (R_f 0.39). The reaction is

-18-

quenched at 0°C by adding 300 mg solid sodium bicarbonate. Water (5 ml) is added and the mixture is extracted with 30 ml methylene chloride. The organic fraction is washed with 15 ml water, dried over MgSO_4 and concentrated under vacuum to give 1.5 g (75%) of crude bromide product. Column chromatography (silica gel, hexane:ethyl acetate, 1:1) gives 1 g pure bromoketone, (mp 129-130°C, Rf 0.47), NMR (CHCl_3) δ 1.33 (d, 3 H, J = 7 Hz), 1.40 (s, 9 H), 3.10 (d, J = 14 Hz), 4.00 (s, 2 H), 4.40 (m, 1 H), 4.81 (m, 1 H), 5.15 (m, 1 H), 6.70 (m, 1 H), 7.45 (m, 5 H).

Example 5

N-Benzyloxycarbonyl-L-alanyl bromomethylketone (Z-Ala-bromomethyl ketone). This bromoketone is made in the manner of the bromoketone of Example 4 except that N-benzyloxycarbonyl-2-carbamoyl-diazoketone is used as a starting material. The following results obtain: 40% yield, Rf 0.48, mp 72-75°C, NMR (CHCl_3) δ 1.43 (d, 3 H, J = 7 Hz), 4.06 (s, 2 H), 4.66 (m, 1 H), 5.12 (s, 2 H), 7.26-7.35 (m, 5 H).

Example 6

N-t-Butyloxycarbonyl L-phenylalanyl-alanine-2-methoxycarbonyl phenoxymethyl ketone. The bromoketone of Example 4 (500 mg, 1.2 mm) is mixed with potassium fluoride (137 mg, 2.4 mm), methyl salicylate 357 mg, 2.3 mm) and 2.8 ml DMF under argon at 35°C for 1 hr. The reaction mixture is diluted in 50 ml ethyl acetate and the organic portion is washed with water (3X25 ml), dried over MgSO_4 and concentrated to 1 g. Purification by column chromatography (silica gel, hexane:ethyl acetate 1:1, Rf 0.465) yields 306 mg N-t-butyloxycarbonyl L-phenylalanyl-alanine-2-methoxycarbonyl phenoxymethyl ketone (56%), mp 102.9-106.6°C. NMR (CDCl_3) δ 1.40 (s, 9 H), 1.45 (d, 3H), 3.09 (m, 1 H), 3.95 (s, 3 H), 4.55 (d, 2 H),

-19-

5.10 (m, 1 H); MS, m/e (485, MH^+).

Example 7

N-BOC-Phe-Ala-phenoxyethyl ketone. This compound is made in the same manner as the compound of Example 6 except
5 that the reagent concentrations are: BOC-Phe-Ala-bromoketone (120 mg, 0.29 mm), potassium fluoride (150 mg, 2.5 mm), phenol (180 mg, 1.9 mm) and DMF (0.5 ml). Reaction yield is 85%; mp 117.5°C; MS m/e (427.30, MH^+); NMR ($CDCl_3$) δ
1.40-1.50 (d, J = 8 Hz), 1.42 (s, 9), 3.10 (d, 2 H), 4.40 (m,
10 1 H), 4.75 (s, 2 H), 4.50-5.50 (m, 4 H), 6.50-7.50 (m, 10H).

Example 8

Z-Ala-phenoxyethyl ketone. This compound is made in the same manner as the compound of Example 6 except that the reagent concentrations are: bromoketone (300 mg, 0.88 mm),
15 potassium fluoride (100 mg, 0.17 mmol), 0.3 ml dry DMF and methyl salicylate (260 mg, 1 mmol). The mixture is then heated under argon for 10 min at 98°C, cooled to 0°C, diluted with 10 ml water and extracted with 5 ml ethyl acetate. The organic layer is washed with 10 ml water, dried over $MgSO_4$,
20 concentrated and purified by TLC (hexane:ethyl acetate, 1:1), silica gel with the product collected at Rf 0.42. Yield after column chromatography is 150 mg (46%). NMR ($CDCl_3$) δ
1.57 (d, 3 H, J = 7 Hz), 4.05 (s, 3 H), 4.66 (s, 2H), 4.92 (m, 1 H), 5.21 (s, 2 H), 6.05 (m, 1 H), 6.60-8.10 (m, 8 H).
25 MS m/e (371, MH^+). Anal. C (63.72), H (5.65), N (4.33); calc. C (64.68), H (5.69), N (3.77).

Example 9

Synthesis of Morpholine carbonyl phenylalanyl
2-aminoisobutyric methyl phenoxy ketone.

30 Synthesis of 2-aminoisobutyric methylester HCl salt. To 2-aminoisobutyric acid in 125 ml of methanol is added 125 ml of methanol saturated with HCl. The initial mixture becomes

-20-

a solution and the reaction is sealed and allowed to stir at room temperature overnight. The next day the reaction is evaporated, first neat and then from toluene to give a solid.

Synthesis of morpholine carbonyl 2-aminoisobutyric acid.

- 5 Morpholine carbonyl phenylalanine (3.6 g, 0.013 mol) in 200 ml of THF is cooled to -20°C under argon. One equivalent of N-methylmorpholine followed by one equivalent of isobutyl chloroformate is added and the reaction is stirred under argon for 10 min. The hydrochloride salt of methylaniline
10 methyl ester is added to the mix, followed by 1.2 equivalents of N-methylmorpholine. The reaction is allowed to come slowly to room temperature and is stirred overnight. The reaction is poured into an equal volume of 1N hydrochloric acid and extracted with ethyl acetate (2x100 ml). The
15 combined extracts are washed with aqueous sodium bicarbonate (2x50 ml) and dried over sodium sulfate. The solvent is evaporated to the methyl ester as a white solid, m.p. 174-188°C.

Conversion to the acid. Morpholine

- 20 carbonyl-2-aminoisobutyric methyl ester (5.65 mmol) is dissolved in 60 ml of methanol containing 6 ml (1N) sodium hydroxide and is stirred under argon overnight. The reaction is then concentrated, diluted with ethyl acetate and extracted with water (50 ml) containing 7 ml 1N sodium
25 hydroxide. The water is neutralized and extracted with ethyl acetate, and is then dried (Na_2SO_4) and concentrated to give a white solid, m.p. 162-166°C.

- The acid is then converted to the 2-carbomethoxy phenoxymethylketone as previously described in Example 2.
30 The relative inhibition of cathepsin B for this inhibitor is 0.2 times that of Z-Phe-Ala-CH₂F.

Example 10

Synthesis of peptidyl methylfluoroketone ketals:
synthesis of the ketal of Mu-Phe-HPhe fluoromethyl ketone.

-21-

Ethylene glycol (25 ml) is dehydrated by heating at 140°C for one hour under a stream of nitrogen. The temperature is then reduced to 70°C and 1.8 g of pTSA was added followed by 310 mg (0.69 mmol) of Mu-Phe-HPhe methylfluoroketone. After 1 hr
5 TLC (CHCl₃:MeOH, 9:1) on silica gel showed a product R_f 0.433 above the starting material R_f 0.396. The reaction is poured into twice its volume of sodium bicarbonate and ice. The resulting mixture is extracted twice with 50 ml ethyl acetate. The combined organic layers are washed with brine,
10 dried over MgSO₄ and concentrated to give a solid foam. The product may be chromatographed (200 g silica gel, CHCl₃:isopropanol, 9:1) to give a high yield of ketal which was shown to be free from the ketone by a single triplet (-221.35 ppm) in the fluorine NMR. (Ketone triplet 220.55
15 ppm).

Example 11

Preparation of a modified arginine derivative at P₁:
synthesis of Mu-Phe-δ-(4,6-dimethyl-2-pyrimidyl) ornithine methyl ester. In a 250 ml round bottom flask was placed
20 1.08 mmol of Mu-Phe-OH in 100 ml THF. The solution was cooled under argon to -20° and one equivalent of N-methylmorpholine and one equivalent of isobutyl chloroformate were added. After 10 min δ-(4,6-dimethyl-2-pyrimidyl) ornithine methyl ester
25 hydrochloride salt (prepared in the manner of Gilbert and Leary, Biochemistry, vol. 14, pp. 5194-99), was added followed by one more equivalent of N-methylmorpholine. The next day the reaction is poured into water and extracted with ethyl acetate. The organic layer was dried (Na₂SO₄) and
30 concentrated to give 130 mg of a white solid. R_f (CHCl₃:isopropanol, 8:2) = 0.43. This material was saponified with one equivalent of 1N sodium hydroxide in the usual way and taken to the phenoxy ketone as in Example 2.

Example 12

Determination of Surprisingly Effective Proteinase Inhibitors. Cathepsin B, purified from human liver, was obtained from Enzyme Systems Products (Dublin, CA). The activity of the enzyme used in this Example was 50 mU per ml at 30°C, in 50 mm sodium phosphate buffer, pH 6.2, 5 mm DTT, 2 mm EDTA, with Z-Leu-Arg-Arg-7-amino-4-trifluoromethyl coumarin (Z-Leu-Arg-Arg-AFC) as substrate.

Cathepsin B assays were done under the following conditions: Five µl of enzyme stock solution was preincubated with 435 µl of phosphate buffer for 30 minutes to activate the enzyme. Nine µl of 20 mm stock substrate solution (in dimethylformamide) were added and mixed with the activated enzyme. Activity was followed by the release of free AFC as monitored by a Perkin-Elmer LS-5B spectrofluorometer (excitation=400nm, emission=505nm).

The inhibitors described herein were prepared in 20 mm stock solutions in DMF. Five µl aliquots of each inhibitor were added to the enzyme 2 min before the substrate was added to the mix. Potency was determined relative to the activity of a standard cathepsin B inhibitor, Z-Phe-Ala-CH₂F (relative potency of 1.0).

It was determined that inhibitors having a side chain on the P₂ amino acid selected from the group consisting of phenylalanyl (Phe), leucyl (Leu), tyrosyl (Tyr), valyl (Val) and substituted analogues thereof, including Tyr(OMe), were particularly effective against cathepsin B. Further, inhibitors with a side chain on the P₁ amino acid selected from the group consisting of alanyl (Ala), arginyl (Arg), histidyl (His), homophenylalanyl (HPhe), phenylalanyl (Phe), ornithyl (Orn), seryl (Ser), threonyl (Thr), thiazoles and amino thiazoles and modifications thereof including 4,6-dimethyl or 4,6-dihydroxy pyrimidyl, and substituted analogues thereof, were also particularly effective. The

-23-

relative potencies of such tested compounds were typically between 2.0 and 10.0, with relative potencies in excess of 25.0 being observed for some compounds. The potencies of members of this relatively small class of inhibitors, those
5 having P₁ and P₂ amino acid side chains selected from the above-described groups, were surprisingly high relative to the potencies of inhibitors with alternative side chains.

Example 13

Treatment of Rheumatoid Arthritis with Mu-Phe-HPhe-O-Ph.

10 DBA/Lac mice were injected with 200 µg of type II chick collagen emulsified in Freund's complete adjuvant on day 0 and on day 21. Mu-Phe-HPhe-O-Ph was suspended in an ethanol solution which was then diluted to 10% (aq) and administered by gavage at a dose of 10 mg/kg/day from day 21 until
15 sacrifice at day 49. The severity of joint inflammation was evaluated grossly at seven day intervals beginning on day 21.

The effect of oral administration of Mu-Phe-HPhe-O-Ph on the severity of bone lesions in adjuvant-induced arthritis
20 was determined by evaluating osseous mineralization, periostat proliferation, bone erosion, joint space narrowing and osseous fragmentation. All lesions were scored on a scale of 0 (normal architecture) to 3 (severe or marked changes). Values calculated were mean values \pm 1 std. error
25 of the mean. The "bone lesion severity" values of Mu-Phe-HPhe-O-Ph treated animals were significantly ($p < 0.05$) lower than the values of control animals for each parameter tested.

The effect of oral administration of Mu-Phe-HPhe-O-Ph on
30 the histological aspects of adjuvant-induced arthritis was determined by evaluating inflammation, focal ulcers, tibiotarsal joint cartilage destruction, bone destruction and periosteal proliferation. Inflammation was scored on a scale of 0 (no inflammation) to 3 (severe) based on the extent of

-24-

edema and cell infiltration. Focal ulcer cartilage destruction was measured as the percent of articular cartilage surfaces with focal destruction which exposed underlying subchondral bone. Tibiotarsal joint cartilage
5 destruction was measured as the percent of articulating surfaces with destruction of the subchondral bone. Values calculated were mean values \pm 1 std. error of the mean. The "histological aspects" values of Mu-Phe-HPhe-O-Ph treated animals were significantly ($p < 0.05$) lower than the values
10 of control animals for each parameter tested.

Example 14

Treatment of Rheumatoid Arthritis with Mu-Leu-HPhe-O-Ph.

DBA/Lac mice were injected with 200 μ g of type II chick
15 collagen emulsified in Complete Freund's Adjuvant on day 0 and on day 21. Mu-Phe-HPhe-O-Ph was suspended in phosphate buffered saline and administered by gavage from day 21 until sacrifice at day 35. Doses of between 3 mg/kg/day and 25
mg/kg/day were used, with the daily dosage being held
20 constant over time for each test. The severity of joint inflammation was evaluated grossly at seven day intervals beginning on day 21.

The effect of oral administration of Mu-Phe-HPhe-O-Ph on the severity of bone lesions in adjuvant-induced arthritis
25 was determined by evaluating osseous mineralization, periostat proliferation, bone erosion, joint space narrowing and osseous fragmentation. All lesions were scored on a scale of 0 (normal architecture) to 3 (severe or marked changes). Values calculated were mean values \pm 1 std. error
30 of the mean. The "bone lesion severity" values of Mu-Phe-HPhe-O-Ph treated animals were significantly ($p < 0.05$) lower than the values of control animals for each parameter tested.

Example 15**Treatment of Rheumatoid Arthritis with**

Mu-Tyr(OMe)-HPhe-O-Ph. Rats were injected with adjuvant on day 0 and were treated with Mu-Tyr(OMe)-HPhe-O-Ph in ground
5 diet from the time of adjuvant injection until sacrifice at day 32. Over the course of the disease, animals were evaluated in terms of a mean clinical score, a derived series of clinically observed parameters normalized to facilitate graphical presentation. Mean paw volumes were also measured
10 using normalized units throughout the course of the disease. At the end of the study, the animal groups were sacrificed and evaluated by X-ray analysis.

The mean clinical scores of Mu-Tyr(OMe)-HPhe-O-Ph treated rats were significantly lower than the scores for untreated
15 animals. Similarly, mean paw volumes were also significantly reduced. This Example demonstrates the beneficial effect of Mu-Tyr(OMe)-HPhe-O-Ph on adjuvant-induced arthritis.

Example 16**Treatment of Osteo Arthritis with Mu-Phe-HPhe-O-Ph.**

20 The skeletons of young growing rats were radioactively labelled by injections with ¹⁴C-tetracycline over a 10 day period. The amount of bone resorption was then determined each day as the amount of radioactivity excreted in the urine. After 10 days of control excretion, test compounds
25 were administered for three days subcutaneously and their effects on the daily excretion of tetracycline were determined. Tested compounds were dissolved in DMSO/PEG at a concentration of 25 mg/ml and were injected once per day subcutaneously in a volume of 2 ml/kg.

30 Mu-Phe-HPhe-O-Ph inhibited bone resorption in vivo as measured by tetracycline excretion. The effect was as great as that of a maximal dose of calcitonin. The onset of inhibition was rapid, with the maximum effect occurring only

-26-

24 hours after dosing commenced.

At the end of the experiment, lesions were visible at the sites of injection. Histological evaluation revealed extensive inflammation of the subcutaneous fat layer
5 extending into the overlying dermis and underlying muscle layers. Tissue necrosis was also observed.

Example 17

Treatment of Osteo Arthritis with Mu-Leu-HPhe-O-Ph.

The skeletons of young growing rats were radioactively
10 labelled by injections with ^{14}C -tetracycline over a 10 day period. The amount of bone resorption was then determined each day as the amount of radioactivity excreted in the urine. After 10 days of control excretion, test compounds
15 were administered for three days subcutaneously and their effects on the daily excretion of tetracycline were determined. Tested compounds were dissolved in DMSO/PEG at a concentration of 25 mg/ml and were injected once per day subcutaneously in a volume of 2 ml/kg.

Mu-Leu-HPhe-O-Ph inhibited bone resorption in vivo as
20 measured by tetracycline excretion. The effect was nearly as great as that of a maximal dose of calcitonin, but was not quite as great as that of Mu-Phe-HPhe-O-Ph. Again, the onset of inhibition was rapid, with the maximum effect occurring only 24 hours after dosing commenced.

25 At the end of the experiment, lesions were visible at the sites of injection. Histological evaluation revealed extensive inflammation of the subcutaneous fat layer extending into the overlying dermis and underlying muscle layers. Tissue necrosis was also observed.

Example 18

Treatment of Osteo Arthritis with Mu-Phe-HPhe-O-Ph.

30 $^{45}\text{Ca}^{2+}$ -labelled bones (calvaria) of neonatal mice were maintained in culture. The effect of the test compound on

-27-

- the rate of parathyroid hormone-stimulated $^{45}\text{Ca}^{2+}$ release was measured over a 24 hour period. An inhibitor of bone resorption (at maximal concentrations) would be expected to inhibit this rate by approximately 70%. The nature of the residual, uninhibitable $^{45}\text{Ca}^{2+}$ -release is not known but could represent chemical exchange of $^{45}\text{Ca}^{2+}$ with the medium. Mu-Phe-HPhe-O-Ph was dissolved in dimethylsulphoxide which was present in the incubation at concentrations < 0.1%.
- 10 Mu-Phe-HPhe-O-Ph inhibited resorption in a concentration-related manner. The shape of the inhibition curve appeared to be biphasic in that 50% of the rate of $^{45}\text{Ca}^{2+}$ -release could be inhibited by 1 μm inhibitor whereas at 100 μm an additional 20% was also blocked.
- 15 IC_{50} values were calculated based upon the initial phase of inhibition.

Example 19

- Treatment of Osteo Arthritis with Mu-Leu-HPhe-O-Ph.**
- $^{45}\text{Ca}^{2+}$ -labelled bones (calvaria) of neonatal mice were maintained in culture. The effect of the test compound on the rate of parathyroid hormone-stimulated $^{45}\text{Ca}^{2+}$ release was measured over a 24 hour period. An inhibitor of bone resorption (at maximal concentrations) would be expected to inhibit this rate by approximately 70%. The nature of the residual, uninhibitable $^{45}\text{Ca}^{2+}$ -release is not known but could represent chemical exchange of $^{45}\text{Ca}^{2+}$ with the medium. Mu-Leu-HPhe-O-Ph was dissolved in dimethylsulphoxide which was present in the incubation at concentrations < 0.1%.
- 20 Mu-Leu-HPhe-O-Ph inhibited resorption in a concentration-related manner. The shape of the inhibition curve appeared to be biphasic in that 50% of the rate of $^{45}\text{Ca}^{2+}$ -release could be inhibited by 1 μm inhibitor whereas at 100 μm an additional 20% was also blocked.
- 30

-28-

IC₅₀ values were calculated based upon the initial phase of inhibition.

Example 20

Treatment of Osteo Arthritis with

5 Mu-Tyr(OMe)-HPhe-O-Ph.

⁴⁵Ca²⁺-labelled bones (calvaria) of neonatal mice were maintained in culture. The effect of the test compound on the rate of parathyroid hormone-stimulated ⁴⁵Ca²⁺ release was measured over a 24 hour period. An inhibitor of bone
10 resorption (at maximal concentrations) would be expected to inhibit this rate by approximately 70%. The nature of the residual, uninhibitable ⁴⁵Ca²⁺-release is not known but could represent chemical exchange of ⁴⁵Ca²⁺ with the medium. Mu-Tyr(OMe)-HPhe-O-Ph was dissolved in
15 dimethylsulphoxide which was present in the incubation at concentrations < 0.1%.

Mu-Tyr(OMe)-HPhe-O-Ph inhibited resorption in a concentration-related manner. The shape of the inhibition curve appeared to be biphasic in that 50% of the rate of
20 ⁴⁵Ca²⁺-release could be inhibited by 1 µm inhibitor whereas at 100 µm an additional 20% was also blocked. IC₅₀ values were calculated based upon the initial phase of inhibition.

Example 21

25 Treatment of Osteo Arthritis with Z-Phe-Ala-O-Ph.

⁴⁵Ca²⁺-labelled bones (calvaria) of neonatal mice were maintained in culture. The effect of the test compound on the rate of parathyroid hormone-stimulated ⁴⁵Ca²⁺ release was measured over a 24 hour period. An inhibitor of bone
30 resorption (at maximal concentrations) would be expected to inhibit this rate by approximately 70%. The nature of the residual, uninhibitable ⁴⁵Ca²⁺-release is not known but could represent chemical exchange of ⁴⁵Ca²⁺ with the

-29-

medium. Z-Phe-Ala-O-Ph was dissolved in dimethylsulphoxide which was present in the incubation at concentrations < 0.1%.

Z-Phe-Ala-O-Ph inhibited resorption in a concentration-related manner. The shape of the inhibition curve appeared to be biphasic in that 50% of the rate of $^{45}\text{Ca}^{2+}$ -release could be inhibited by 1 μm inhibitor whereas at 100 μm an additional 20% was also blocked. IC_{50} values were calculated based upon the initial phase of inhibition.

Example 22

Treatment of Osteo Arthritis with

Mu-Tyr(OMe)-HPhe-O-Ph.

$^{45}\text{Ca}^{2+}$ -labelled bones (calvaria) of neonatal mice were maintained in culture. The effect of the test compound on the rate of parathyroid hormone-stimulated $^{45}\text{Ca}^{2+}$ release was measured over a 24 hour period. An inhibitor of bone resorption (at maximal concentrations) would be expected to inhibit this rate by approximately 70%. The nature of the residual, uninhibitable $^{45}\text{Ca}^{2+}$ -release is not known but could represent chemical exchange of $^{45}\text{Ca}^{2+}$ with the medium. Mu-Tyr(OMe)-HPhe-O-Ph was dissolved in dimethylsulphoxide which was present in the incubation at concentrations < 0.1%.

Mu-Tyr(OMe)-HPhe-O-Ph was found to inhibit resorption in mouse calvaria with an IC_{50} of approximately 40 nm. The shape of the inhibition curve was not simple (as was observed with other cysteine protease inhibitors) and the IC_{50} value is for the initial phase of inhibition.

Example 23

Treatment of Malaria with Mu-Phe-HPhe-O-Ph. Soluble extracts of *P. falciparum* and *P. vinckei* were prepared and proteolytic activity was assayed with fluorogenic peptide

-30-

substrate Z-Phe-Arg-AFC. The activity of control samples was compared with activities in the presence of multiple concentrations of Mu-Phe-HPhe-O-Ph. The concentration at which activity was inhibited by 50% (IC_{50}) was calculated.

5 Mu-Phe-HPhe-O-Ph inhibited the activity of both *P. falciparum* and *P. vinckei*. IC_{50} values of 3.0 nm and 5.1 nm, respectively, obtain.

Example 24

Treatment of Malaria with Mu-Leu-HPhe-O-Ph. Soluble
10 extracts of *P. falciparum* and *P. vinckei* were prepared and proteolytic activity was assayed with fluorogenic peptide substrate Z-Phe-Arg-AFC. The activity of control samples was compared with activities in the presence of multiple concentrations of Mu-Leu-HPhe-O-Ph. The concentration at
15 which activity was inhibited by 50% (IC_{50}) was calculated.

Mu-Leu-HPhe-O-Ph inhibited the activity of both *P. falciparum* and *P. vinckei*. IC_{50} values of 0.42 nm and 15 nm, respectively, obtain.

Example 25

20 Treatment of Malaria with Mu-Phe-Arg(NO_2)-O-Ph. Soluble extracts of *P. falciparum* and *P. vinckei* were prepared and proteolytic activity was assayed with fluorogenic peptide substrate Z-Phe-Arg-AFC. The activity of control samples was compared with activities in the presence
25 of multiple concentrations of Mu-Phe-Arg(NO_2)-O-Ph. The concentration at which activity was inhibited by 50% (IC_{50}) was calculated.

Mu-Phe-Arg(NO_2)-O-Ph inhibited the activity of both *P. falciparum* and *P. vinckei*. IC_{50} values of 2.4 nm and 110
30 nm, respectively, obtain.

Example 26

Treatment of Malaria with Mu-Tyr(OCH_3)₂-HPhe-O-Ph.

-31-

Soluble extracts of *P. falciparum* and *P. vinckei* were prepared and proteolytic activity was assayed with fluorogenic peptide substrate Z-Phe-Arg-AFC. The activity of control samples was compared with activities in the presence of multiple concentrations of Mu-Tyr(OCH₃)₂-HPhe-O-Ph. The concentration at which activity was inhibited by 50% (IC₅₀) was calculated.

Mu-Tyr(OCH₃)₂-HPhe-O-Ph inhibited the activity of both *P. falciparum* and *P. vinckei*. IC₅₀ values of 8.5 nm and 18 nm, respectively, obtain.

Example 27

Treatment of Malaria with Mu-Phe-HPhe-methyl salicylate. Soluble extracts of *P. falciparum* were prepared and proteolytic activity was assayed with fluorogenic peptide substrate Z-Phe-Arg-AFC as described in Rosenthal, Wollish, Palmer and Rasnick, "Antimalarial Effects of Peptide Inhibitors of a Plasmodium falciparum Cysteine Proteinase," 88 J. Clin. Invest. 1467. The activity of control samples was compared with activities in the presence of multiple concentrations of Mu-Phe-HPhe-methyl salicylate. The concentration at which activity was inhibited by 50% (IC₅₀) was calculated.

Mu-Phe-HPhe-methyl salicylate inhibited the activity of *P. falciparum*. An IC₅₀ value of 42 nm obtains.

25

Example 28

Treatment of Gingivitis with Mu-Phe-HPhe-O-Ph. The effect of the inhibitor on gingival inflammation was studied by investigating its ability to prevent the development of experimental gingivitis. Twenty human subjects with healthy gingiva and no periodontal disease were recruited. The subjects received oral hygiene instruction and scaling prior to baseline. The test sites were the mesibuccal crevices on the upper first and second molars and premolars (bicuspid).

-32-

Acrylic shields were made to cover the gingival margins of these teeth and were worn during oral hygiene to prevent brushing of the test and control sites. During the test period of three weeks the subjects were told to brush only the lower teeth and upper anteriors and to wear the shields during brushing. The left side was used as the test side and the right was the control side in 10 subjects. The sides were reversed in the other 10 subjects so the subjects could act as their own controls. Thirty second GCF samples were taken prior to clinical measurements at baseline and at 1, 2, 3 and 4 weeks. These were assayed for cathepsins B and L-like activities. Clinical measurements of gingival index (GI), gingival bleeding index (GBI) and plaque index (PLI) were taken at test and control sites at zero, one, two, three and four weeks. Following the baseline measurements the inhibitor and placebo were placed at test and control sites and sealed in with Coe-pak for a week. The inhibitor and placebo were coded so that the study was blind.

The GI, GBI and PLI and cysteine proteinase levels were compared at test and control sites. It was observed that the use of Mu-Phe-HPhe-O-Ph reduced all parameters tested at the test sites. Mu-Phe-HPhe-O-Ph can therefore be seen to be effective in treating periodontal diseases such as gingivitis.

The foregoing Examples demonstrate the use of the disclosed proteinase inhibitors to treat specific medical conditions including rheumatoid arthritis, osteo arthritis, malaria and gingivitis are disclosed. The surprising efficacy of the compounds in such in vivo applications could not have been predicted from the prior art, and was determined only after extensive experimentation.

It is understood that a number of variations may be made to adapt the present invention to a particular medical condition without changing the basic compositions and methods disclosed herein. Therefore, while the invention has been

-33-

illustrated and described in detail in the foregoing
Examples, the same are to be considered illustrative and not
restrictive in character. It is to be understood that the
preferred embodiments have been shown and described, and that
5 all changes and modifications that come within the spirit of
the invention are desired to be protected.

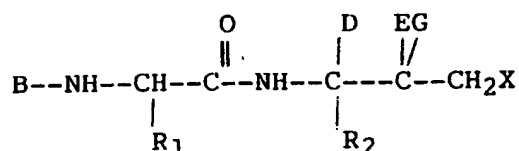
-34-

CLAIMS

We claim:

1. A method of treating medical conditions characterized by extralysosomal cathepsin B and/or cathepsin L, comprising the steps of:

(a) providing a compound of the formula:



10 wherein

B is H or an amino acid blocking group for the N-terminal amino acid nitrogen;

15 R_1 is an optionally protected α -amino acid side chain on the P_2 amino acid residue, and is selected such that the P_2 amino acid residue is the residue of an α -amino acid selected from the group consisting of phenylalanine (Phe), leucine (Leu), tyrosine (Tyr) and valine (Val), and substituted analogues thereof, particularly including Tyr(OMe);

20 R_2 is an optionally protected α -amino acid side chain on the P_1 amino acid residue, and is selected such that the P_1 amino acid residue is the residue of an α -amino acid selected from the group consisting of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His), homophenylalanine (HPhe), phenylalanine (Phe), ornithine (Orn), serine (Ser) and threonine (Thr), and substituted analogues thereof;

30 X is a fluorine-free leaving group selected from the group consisting of phenoxy, substituted phenoxy and heterophenoxy;

E and G are atoms more electronegative than carbon,

-35-

wherein E and G may be the same atom, as in a carbonyl or hydrazone, bonded to the peptide chain by a double bond, or two separate atoms each bonded to the peptide chain by a single bond; and

- 5 D is hydrogen, methyl or a substituted methyl;
or a pharmaceutically acceptable salt thereof; and
(b) delivering said compound to a medical patient.

2. The method of claim 1 wherein said compound is delivered to a medical patient in a pharmaceutically acceptable
10 carrier.

3. A method according to claim 1 wherein said medical condition characterized by extralysosomal cathepsin B and/or cathepsin L activity is rheumatoid arthritis.

4. A method according to claim 1 wherein said medical
15 condition characterized by extralysosomal cathepsin B and/or cathepsin L activity is osteo arthritis.

5. A method according to claim 1 wherein said medical condition characterized by extralysosomal cathepsin B and/or cathepsin L activity is malaria.

20 6. A method according to claim 1 wherein said medical condition characterized by extralysosomal cathepsin B and/or cathepsin L activity is a periodontal disease.

7. A method according to claim 1 wherein R_2 is an optionally blocked side chain on the P_1 amino acid residue
25 such that the P_1 amino acid residue is the residue of homophenylalanine.

8. A method according to claim 1 wherein B is a 4-morpholinylcarbonyl blocking group.

-36-

9. A method according to claim 8 wherein R_2 is an optionally blocked side chain on the P_1 amino acid residue such that the P_1 amino acid residue is the residue of homophenylalanine.

5 10. A method according to claim 8 wherein R_2 is an optionally blocked heterocyclic analogue of the residue of an α -amino acid selected from the group consisting of alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), histidine (His), homophenylalanine (HPhe),
10 phenylalanine (Phe), ornithine (Orn), serine (Ser) and threonine (Thr), particularly including thiazoles and amino thiazoles.

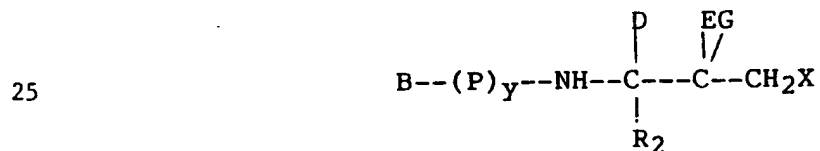
11. A method according to claim 1 wherein X is a fluorine-free leaving group which is also bonded back to
15 R_2 .

12. A method according to claim 1 wherein D is methyl or a substituted methyl.

13. A method according to claim 1 wherein X is a heterocyclic leaving group.

20 14. A method according to claim 1, and having a hydrazine replacement for the P_1 nitrogen.

15. A cathepsin B and/or cathepsin L inhibitor of the formula:



-37-

wherein

- B is a 4-morpholinylcarbonyl blocking group;
each $(P)_y$ is an optionally protected α -amino acid residue;
5 y is a number between zero and three; and
 R_2 is a side chain on the P_1 amino acid residue such that the P_1 amino acid residue is the residue of homophenylalanine;
X is a leaving group;
10 E and G are atoms more electronegative than carbon, wherein E and G may be the same atom, as in a carbonyl or hydrazone, bonded to the peptide chain by a double bond, or two separate atoms each bonded to the peptide chain by a single bond; and
15 D is hydrogen, methyl or a substituted methyl; or a pharmaceutically acceptable salt thereof.
16. A cathepsin B and/or cathepsin L inhibitor according to claim 15 wherein X is a fluorine-free leaving group which is also bonded back to R_2 .
- 20 17. A cathepsin B and/or cathepsin L inhibitor according to claim 15 wherein D is methyl or a substituted methyl.
18. A cathepsin B and/or cathepsin L inhibitor according to claim 15 wherein X is a heterocyclic leaving group.
19. A cathepsin B and/or cathepsin L inhibitor according to
25 claim 15, and having a hydrazine replacement for the P_1 nitrogen.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/07767

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/00,37/02; C07C 229/06,381/02; C07D 265/30

US CL :514/17,18,19; 560/10,18,37,45; 530/330,331,332; 544/168

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/17,18,19; 560/10,18,37,45; 530/330,331,332; 544/168

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CHEMICAL ABSTRACTS

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A, 4,518,528 (RASNICK) 21 MAY 1985, ENTIRE DOCUMENT.	1-19
X	US,A, 5,055,451 (KRANTZ ET AL) 08 OCTOBER, ENTIRE DOCUMENT.	1-19
A	EP,A, 0,272,671 (KRANTZ ET AL) 29 JUNE 1988, ENTIRE DOCUMENT.	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 DECEMBER 1993

Date of mailing of the international search report

10 DEC 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

FLOYD HIGEL

Telephone No. (703) 308-1235